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Teratogen Metabolism: Thalidomide Activation is Mediated by Cytochrome P-450

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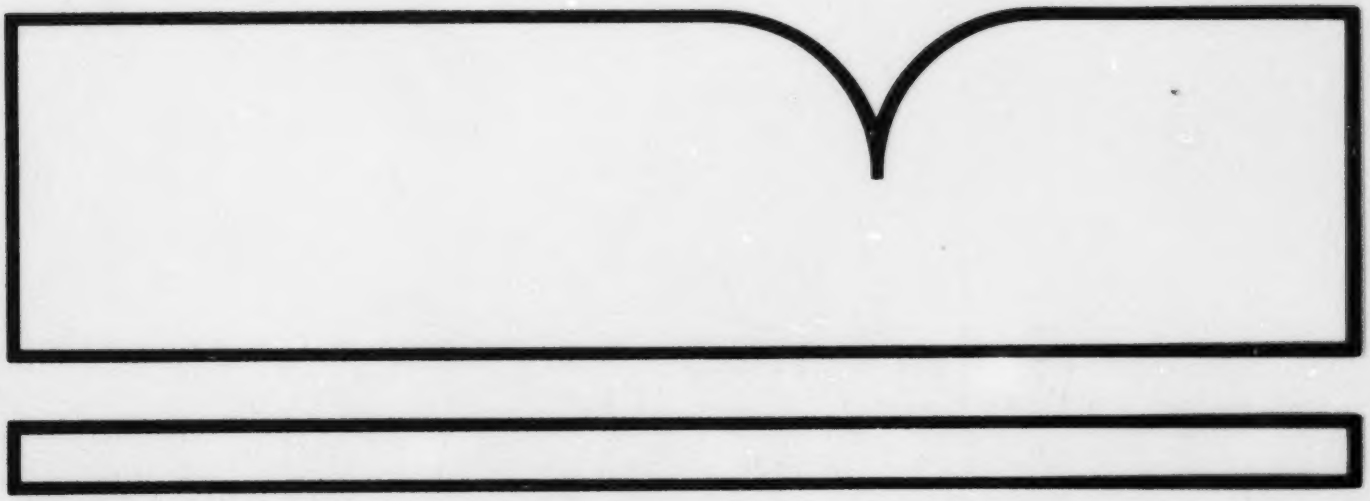

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16. ABSTRACT

A metabolite of thalidomide generated by hepatic microsomes inhibited the attachment of tumor cells to concanavalin A-coated polyethylene. Evidence that metabolite formation is mediated by microsomal cytochrome *P*-450 is presented. Microsomes incubated with thalidomide underwent a type I spectral shift. Metabolite formation was reduced or eliminated by carbon monoxide, SKF-525A, metyrapone, and *N*-octylamine. Superoxide dismutase treatment had no effect. Metabolite formation required microsomes and NADPH and was dependent on the length of 37°C incubation. The metabolite could be isolated by successive hexane and chloroform extractions. It is likely the inhibitory thalidomide metabolite was generated by a minor cytochrome *P*-450 species. Whether this thalidomide metabolite is involved in the drug's teratogenic activity remains to be shown.

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SHORT COMMUNICATION

Teratogen Metabolism: Thalidomide Activation Is Mediated by Cytochrome *P*-450

Teratogen Metabolism: Thalidomide Activation Is Mediated by Cytochrome *P*-450. BRAUN, A. G., HARDING, F. A., AND WEINREB, S. L. (1986). *Toxicol. Appl. Pharmacol.* **82,** 175–179. A metabolite of thalidomide generated by hepatic microsomes inhibited the attachment of tumor cells to concanavalin A-coated polyethylene. Evidence that metabolite formation is mediated by microsomal cytochrome *P*-450 is presented. Microsomes incubated with thalidomide underwent a type I spectral shift. Metabolite formation was reduced or eliminated by carbon monoxide, SKF-525A, metyrapone, and *N*-octylamine. Superoxide dismutase treatment had no effect. Metabolite formation required microsomes and NADPH and was dependent on the length of 37°C incubation. The metabolite could be isolated by successive hexane and chloroform extractions. It is likely the inhibitory thalidomide metabolite was generated by a minor cytochrome *P*-450 species. Whether this thalidomide metabolite is involved in the drug's teratogenic activity remains to be shown. © 1986 Academic Press, Inc.

While most agents classified as teratogens act only at maternally toxic amounts (Khera, 1984), there are a substantial number of selectively embryotoxic chemicals. These agents are of particular interest since they may alter normal embryonic morphogenic processes without major effects on adult tissues. Perhaps the best known such agent is the infamous sedative thalidomide.

Because of its low solubility thalidomide has low acute oral toxicity in humans and laboratory animals. However at maternal doses of less than 1 mg/kg, severe malformations in most organs are produced (Runner, 1967; Fabro, 1981). Thalidomide is thus prototypical of the most dangerous form of chemical teratogen: one with no maternal toxicity but with devastating toxicity to the embryo.

Efforts to understand the mechanism of thalidomide teratogenesis have been unsuccessful despite 25 years of research. The drug is not a mutagen (Gordon and Blake, 1978); it does not affect cell division or growth in culture and has no detectable effect on the kinetics of numerous enzymes (Keberle *et al.*, 1965).

Several years ago we noted that several salient features of mouse ovarian tumor (MOT) cell attachment to lectin-coated surfaces were shared by embryonic cell-to-cell interactions. Both processes require an intact cytoskeleton, involve cell surface carbohydrates, and require metabolic energy (Edelman *et al.*, 1971; Hausman and Moscona, 1975; Rutishauser *et al.*, 1976; Braun *et al.*, 1982). We therefore hypothesized that teratogens which interfere with embryonic cell interactions may also intefere with lectin-mediated attachment. If this were the case, the attachment system could be used as a simple and rapid *in vitro* assay for this class of chemical teratogens. Thus an assay was developed in which murine ascites tumor (MOT) cells were treated with a test agent and then permitted to sediment onto polyethylene disks coated with concanavalin A. An agent was considered to be potentially capable of interfering with cell interactions if it inhibited attachment at noncytotoxic concentrations. Studies with over 100 chemicals indicated that more than 90% of agents inhibiting attachment *in vitro* were teratogens and that there was a rough quantitative correspondence between inhibitory drug concentration and teratogenic dose in animals (Braun *et al.*, 1982).

While thalidomide did not inhibit attachment, an inhibitory product was formed on incubation with NADPH and hepatic microsomes from uninduced mice, rats, or dogs (Braun and Dailey, 1981; Braun and Weinreb, 1984). Since the production of inhibitory

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product was dependent on microsomes, NADPH, and oxygen, it appeared likely that the enzymes involved were cytochrome *P*-450 isozymes. One difficulty with this possibility was the fact that hepatic microsomes from rats induced with Aroclor 1254, methylcholanthrene, or phenobarbital and mice pretreated with thalidomide were less capable of thalidomide activation than were those from uninduced animals (Braun and Weinreb, 1984; unpublished observations). Thus it appeared as if the activation pathway were either not catalyzed by inducible cytochrome *P*-450 isozymes or, perhaps, were not mediated by *P*-450[1] at all. We therefore wished to obtain further evidence on the role of cytochrome *P*-450 metabolism in thalidomide activation.

In this communication we report that a variety of well-established *P*-450 inhibitors prevent thalidomide activation and conclude activation is mediated by this enzyme system.

METHODS

Thalidomide activation. Thalidomide was incubated with canine hepatic microsomes, NADPH, and tritium-labeled mouse ovarian ascites tumor (MOT) cells for 1 hr and attachment measured as described (Braun and Dailey, 1981; Braun and Weinreb, 1984).

Spectral difference measurements were made in a Perkin–Elmer 533 double-beam scanning spectrophotometer with divided 3-ml cuvettes. In both cuvettes one of the half chambers contained microsomes at 0.3 mg/ml in PBS, the other 0.4 mg/ml thalidomide 1% DMSO-PBS. A baseline difference spectrum taken through both chambers of each cell was measured. The contents of the two halves of one cell were mixed to yield final microsome and thalidomide concentrations of 0.15 and 0.2 mg/ml, respectively. A difference spectrum was then taken using the cell with still separated components as a reference. Similar results were obtained in an Hewlett–Packard HP 8450A digital spectrophotometer. In both cases wavelength calibration was confirmed by a holmium oxide standard.

RESULTS

Our objective in these experiments was to determine whether the *in vitro* metabolic activation of thalidomide to a product which inhibited lectin-mediated cell attachment was catalyzed by cytochrome *P*-450. If microsomal *P*-450 were involved, it was expected that a shift in the *P*-450 spectrum should be induced upon drug addition. When canine hepatic microsomes were mixed with thalidomide, a barely detectable spectral change similar to the cytochrome *P*-450 type I difference spectrum was observed (Fig. 1). However, the characteristic decline in absorptance had its maximum consistently at 416 nm rather than at 420 nm where it is found in most type I difference spectra. Whether this indicates a novel *P*-450 isozyme or a peculiarity of the interaction with thalidomide remains to be determined.

While binding of the drug substrate to the hemoprotein is required for *P*-450-mediated metabolism, the fact of binding does not prove that *P*-450 is involved in the metabolism of inhibitory products. It is possible that *P*-450 catalyzed metabolism produces inactive products and that another microsomal system is involved in cell attachment inhibitor formation. We therefore studied the effects of a series of standard *P*-450 inhibitors on metabolite formation to gain further insight into whether

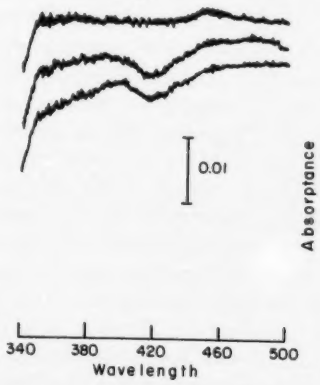

FIG. 1. Optical difference spectrum caused by the addition of thalidomide to canine hepatic microsomes. Details are described under Methods. Upper curve: baseline before mixing. Middle curve: Differences spectrum taken immeidately after mixing. Lower curve: Difference spectrum 20 min after mixing.

[1] Abbreviations used: *P*-450, for convenience we refer to cytochrome P-450 isozymes as *P*-450; MOT, mouse ovarian ascites tumor; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

P-450 was involved in thalidomide activation. Initial experiments examined the effect of carbon monoxide. Thalidomide, microsomes, cofactors, and MOT cells were incubated in room atmosphere or a 1:1 oxygen–nitrogen mixture and yielded metabolite concentrations which inhibited attachment by 50% (Fig. 2). However, when carbon monoxide was substituted for nitrogen in the mixture, almost no inhibitory metabolite(s) was produced (<10% inhibition), and it appeared that the production of a thalidomide metabolite which inhibited attachment involved a carbon monoxide sensitive enzyme.

Three drugs known to inhibit *P*-450 activity, metyrapone, SKF 525A, and *N*-octylamine, were applied *in vitro* and found to inhibit activation. The effect of *N*-octylamine is shown in Fig. 3. Drug concentrations used in the present study were comparable to those required to inhibit *P*-450 activity in other metabolizing systems (Sladel and Mannering, 1969).

The role of active oxygen species in the formation of metabolite(s) that inhibited MOT cell attachment was examined by including superoxide dismutase and/or catalase in the activation mixture. While superoxide dismutase alone did not prevent hepatic microsomes from producing an inhibitory thalidomide metabolite, catalase interfered with metabolite formation (Fig. 4).

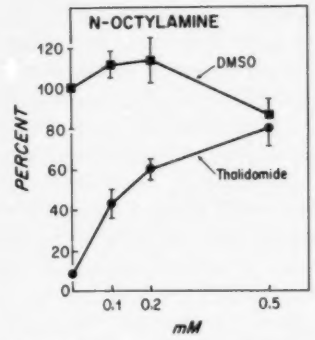

FIG. 3. Effect of *N*-octylamine on thalidomide activation *in vitro*. MOT cells, thalidomide at 500 μg/ml or 1% DMSO vehicle, canine microsomes, cofactors, and the indicated concentration of *N*-octylamine were incubated for 1 hr, and the cell's ability to attach to concanavalin A-coated polyethylene was tested as described in Braun and Weinreb (1984). SEM estimate from three lectin-coated disks indicated. Symbols (●): 500 μg/ml thalidomide; (■): 1% DMSO.

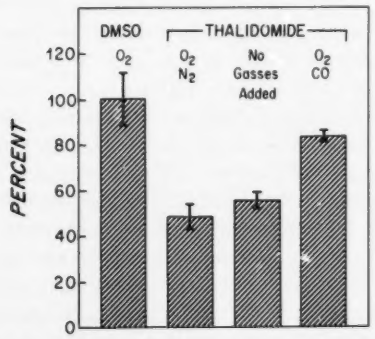

FIG. 2. Effect of carbon monoxide on thalidomide activation. Duplicate 1-ml samples of canine microsomes, tritium-labeled MOT cells, cofactors, and thalidomide at 500 μg/ml in 1% DMSO or 1% DMSO alone were incubated in a sealed container at 37°C for 1 hr following the gas treatment indicated. Cell attachment to three concanavalin A-coated polyethylene disks was then determined. Each bar shows the average percent attachment ± SEM from six lectin-coated disks (three from each replicate sample) under the treatment conditions specified.

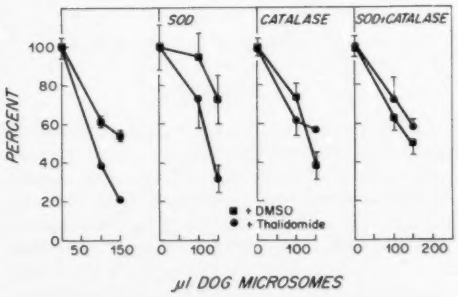

FIG. 4. Effects of catalase and superoxide dismutase on thalidomide activation *in vitro*. Tritium-labeled MOT cells, thalidomide at 500 μg/ml in 1% DMSO or 1% DMSO alone, 50 and 100 μl canine microsomes (at 12 mg protein/ml), NADPH, and 30 μg enzyme/ml were incubated for 1 hr at 37°C. The cells' ability to attach to concanavalin A-coated polyethylene was then determined. Each point is based on a single assay mixture whose inhibitory acitivity was measured by pouring the contents over three lectin-coated disks. The average percentage of control attachment and SEM from these three lectin-coated disks are indicated. Independent, replicate, experiments yielded the same results. Symbols (●): 500 μg/ml thalidomide; (■): 1% DMSO.

The possibility remained that the inhibition described so far was due to an artifact of our complex assay system. If metabolites were involved, it might be possible to isolate the compounds and subsequently demonstrate their inhibitory activity. As a first step toward metabolite purification, microsomes, thalidomide, and NADPH were incubated without MOT cells for 60 min, extracted with hexane to remove microsomal lipids, and then extracted with chloroform to isolate thalidomide and its activation product. The hexane and chloroform extracts were dried under nitrogen, dissolved in DMSO, and tested for their ability to inhibit attachment. Inhibitory thalidomide-derived metabolite was found in the chloroform extract, while nonspecific MOT cell attachment inhibitory activity was confined to the hexane extract (Fig. 5).

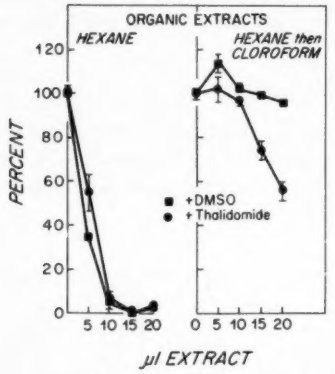

FIG. 5. Isolation of attachment inhibiting material formed from thalidomide *in vitro* by solvent extraction. Thalidomide at 500 µg/ml or 1% DMSO vehicle, canine microsomes at 150 µl/ml (protein concentrations: 12 mg/ml), 0.25 mM NADPH, and 0.05 M sodium phosphate buffer, pH 7.4, in a total volume of 10 ml were incubated at 37°C for 1 hr. To retard spontaneous alkaline hydrolysis (Schumacher *et al.*, 1965), 0.2 ml 0.5 M monobasic sodium phosphate was added to yield a final pH of 6.5. The mixture was extracted with 20 ml hexane and the aqueous layer further extracted with 20 ml chloroform. The organic layers were dried separately under a stream of nitrogen and the residue dissolved in 100 µl DMSO. This extract was tested for its ability to inhibit tumor cell attachment. Tritium-labeled cells were incubated with the indicated volume of extract for 30 min at 37°C, and attachment was determined. Left panel: Hexane extract. Right panel: Chloroform extract from aqueous layer following hexane extraction. Symbols (●): original incubation mixture contained 500 µg/ml thalidomide; (■): original mixture contained 1% DMSO vehicle.

DISCUSSION

The results presented here indicate that the thalidomide metabolite which inhibits lectin-mediated attachment *in vitro* is generated by hepatic microsomal *P*-450. It is possible that the thalidomide metabolite detected in the attachment assay system may cause malformations *in vivo* if present at sufficiently high concentrations in embryonic tissues. Whether this metabolite is actually present in the embryo following maternal oral thalidomide adminstration awaits identification of the metabolite and the development of sensitive techniques for its detection in embryonic tissues.

If a cytochrome *P*-450 generated thalidomide metabolite is responsible for thalidomide teratogenesis, then agents which inhibit *P*-450 function should reduce the drug's teratogenic activity *in vivo*. Thus NZW rabbit embryos should be protected from thalidomide teratogenesis by maternal treatment with SKF 525A. SKF 525A administration has been shown to protect embryonic mice from the teratogenic action of meclizine and chlorcyclizine (Posner *et al.*, 1967).

Two lines of evidence suggest that the *P*-450 isozyme involved in thalidomide activation to a MOT cell attachment inhibiting material may not have been previously described. First, the spectral shift upon thalidomide addition displays a minimum at 416 nm rather than the commonly observed 420 nm position (Fig. 1). Second, hepatic microsomes from rats pretreated, *in vivo*, with drugs that increase the amounts of several *P*-450 isozymes were no more capable of generating an inhibitory metabolite than were hepatic microsomes from untreated animals (Braun and Weinreb, 1984). In addition the small size of the spectral shift in the presence of high thalidomide concentrations suggests that the isozyme may be a minor species in uninduced microsomes.

Catalase prevented inhibitory metabolite formation. While it is possible a peroxide intermediate is involved in thalidomide activation, it is more likely that the catalase interferes with *P*-450 function. The intermediate *P*-450-Fe(III)O_2-substrate complex is in equilibrium with a peroxide form susceptible to catalase decomposition (Mannering, 1981). Thus the presence of catalase in the incubation mixture forces the *P*-450 cycle into a dead end, and substrate oxidation is prevented.

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